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Radioimmunoassay for circulating human guanylin

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Abstract

A highly specific and sensitive radioimmunoassay for circulating human guanylin (guanylin-22–115) has been developed. Antibodies were raised against the amino-terminus (positions 4–16) of the peptide. Western blot analysis confirmed that the antibody selected for radioimmunoassay recognizes circulating high molecular weight (10.3 kDa) guanylin. Extraction and purification of guanylin from blood hemofiltrate and from blood plasma showed that circulating guanylin is detectable in corresponding amounts by the radioimmunoassay and by a specific bioassay. In 30 healthy subjects, the mean plasma concentration of immunoreactive (IR) guanylin was 42 ± 3 fmol/ml. In 22 patients with chronic renal insufficiency, the concentrations of IR-guanylin were significantly enhanced (1,074 ± 24 fmol/ml), indicating that kidneys metabolize and/or eliminate the circulating hormone.

Key words: Guanylin; Radioimmunoassay; Human plasma; Urine; Blood hemofiltrate; Dialysis

1. Introduction

Guanylin is an endogenous hormone that activates intestinal guanylate cyclase (GC-C). It was initially isolated and characterized from rat jejunum as a 15 amino acid peptide [1]. Later it was found that guanylin circulates as a 94 amino acid peptide in human blood [2]. cDNA sequencing revealed that both peptides derive from a larger prohormone of 115 amino acids [3]: the rat peptide is the C-terminal end (positions 101–115) and the circulating human peptide corresponds to positions 22–115 of proguanylin. Synthetic guanylin-101–115 increases cyclic GMP content and stimulates chloride secretion in cultured intestinal epithelial (T84) cells and isolated rat intestinal mucosa [3,4]. These observations have led to the hypothesis that guanylin is involved in the control of intestinal fluid and electrolyte transport. Human guanylin-22–115 also increases cyclic GMP content in T84 cells and stimulates chloride secretion by human intestinal mucosa in vitro, suggesting that the peptide circulating in human blood is a bioactive hormonal form of guanylin and not merely a precursor.

Immunocytochemical studies with antibodies directed against different epitopes of guanylin-22–115 have shown that enterochromaffin (EC) cells are the cellular source of this hormone in the gastrointestinal tract [5]. This suggests that, in the gut, guanylin is a paracrine modulator of electrolyte transport by neighboring enterocytes. It is still not known whether plasma guanylin forms part of an endocrine pathway or whether it accompanies intestinal paracrine secretion. As a modulator of intestinal chloride transport, guanylin may be involved in the pathophysiology of several gastrointestinal diseases, especially those with secretory diarrhea. Measurement of guanylin levels in plasma is important for a better understanding of the functional role of the guanylin system. Therefore, we have developed a radioimmunoassay for the measurement of circulating guanylin-22–115 in human plasma and initiated studies to evaluate the levels of immunoreactive (IR) guanylin in healthy subjects and patients with chronic renal failure.

2. Materials and methods

2.1. Peptide synthesis

Starting from the sequence of the circulating form of human guanylin [2] the following peptides were synthesized: guanylin-25–37 (Gln-Asp-Gly-Asn-Phe-Ser-Phe-Ser-Leu-Glu-Ser-Val-Lys) and (Tyr^{24})-guanylin-25–37. This sequence corresponds to a sequence near the amino-terminus (positions 4–16) of the circulating peptide. The peptides were synthesized on a Zinsser SMPS 350 automated peptide synthesizer using standard Fmoc chemistry [6]. Peptide chains were assembled by TBTU/ DIPEA/HOBT activation on preloaded Wang resins. Guanylin-25–37 was synthesized as a linear peptide and as octameric multiple antigenic peptide (MAP; for immunization) [7]: MAP consists of a core matrix with a branched heptalysine containing eight dendritic chains of guanylin-25–37. Rat guanylin-101–115 was synthesized on a 9050 peptide synthesizer (Millipore, Erkrath, FRG) on Fmoc-Cys(Trt)-PEG-PS-resin. A cystine bridge between residues 107 and 115 was introduced by oxidation with potassium ferricyanide (III), and a cystine bridge between residues 104 and 112 by oxidation with iodine in 5% acetic acid. The purity and sequence of the peptides were checked by reverse phase (RP)-HPLC, capillary zone electrophoresis, mass spectrometry, and automated Edman degradation.

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2.2. Immunization

Guanylin-25–37 (MAP) was dissolved in saline (1 mg/ml) and emulsified with an equal volume of complete Freund's adjuvant (Sigma, Deisenhofen, Germany). 1 ml emulsion (equivalent to 0.5 mg MAP) was injected subcutaneously at multiple sites on the back of 5 New Zealand white rabbits. At intervals of 4 weeks, animals were re-injected with 0.25 mg MAP emulsified in incomplete Freund's adjuvant (Sigma). Blood for testing was withdrawn 14 days after each booster injection. An enzyme-linked immunosorbent assay (ELISA) was used to test antisera for their ability to react with guanylin-25–37 (monomeric peptide). The antiserum with the highest titer (K39) was subsequently used in this study.

2.3. Preparation of [^{125}I](Tyr)-guanylin-25–37

(Tyr^{24})-guanylin-25–37 was radioiodinated by Immundiagnostik (Bensheim, Germany) using the chloramin T method [8]. 1 mCi ^{125}I in a volume of 10 μl was added to 10 μg (Tyr^{24})-guanylin-25–37 in 50 μl of 0.05 M sodium phosphate buffer, pH 7.4. The reaction was initiated by the addition of 10 μl chloramin T solution (20 μg in phosphate buffer). The mixture was shaken for 30 s and the reaction stopped by the addition of sodium metabisulfite solution (20 μg in 10 μl phosphate buffer). Sep-Pak C18 cartridges (Waters; Millipore) were used to separate labelled peptide from free ^{125}I. Specific activity was about 700 μCi/μg.

2.4. Radioimmunoassay procedure

The assay buffer for RIA was 0.01 M phosphate buffer, pH 7.4, containing 0.14 M NaCl, 0.01 M EDTA-dipotassium salt (K_2EDTA), 0.001 M sodium acide. 0.1 mg/ml bovine serum albumin (fraction V; Sigma) and 0.1% Triton X-100. This assay buffer was used to dissolve all reagents and samples. The incubation mixture consisted of 0.1 ml sample or standard (guanylin-25–37, monomeric peptide) and 0.1 ml K39 antiserum (final dilution, 1:3000). Incubation was carried out for 24 h, followed by addition of ^{125}I(Tyr^{24})-guanylin-25–37 (20,000 cpm in 0.1 ml) and further incubation for 24 h. The bound and free ligands were separated by incubation with 0.25 ml goat anti-rabbit γ-globulin (Immundiagnostik) with 5% polyethylene glycol (M_W 6,000) for 1 h, and centrifugation at 2,500 × g for 20 min. The radioactivity in the precipitate was counted in a γ-spectrometer. All procedures were performed at 4°C. The standard curve was calculated using B/B_0 vs. fmol of guanylin-25–37 (standard), see legend to Fig. 1. The amount of guanylin in the unknown samples was extrapolated from the standard curve linearized by a logit-log transformation.

2.5. Purification of circulating human guanylin by RP-HPLC

As a larger source for guanylin we used hemofiltrate collected from patients with chronic renal failure [9]. For purification of guanylin an almost identical procedure as for its isolation was used (for details, see [2]). In brief, hemofiltrate polypeptides were extracted with alginic acid according to the Mutt–Forssmann method [10,11] and the extract corresponding to 3.4 l hemofiltrate was applied to a preparative RP C4 column (300 Å, 20–45 μm, 30 × 125 mm; Parcosil; Biotek, Heidelberg, Germany). The absorbed peptides were eluted with a flow rate of 5 ml/min and fractionated with the following gradient: 0.01 N hydrochloric acid (HCl) to 50% 2-propanol/30% methanol/0.01 N HCl in 60 min; 2-min fractions (step 1). Aliquots of each fraction were tested in the bioassay with T84 cells as well as in the guanylin radioimmunoassay. Fractions containing bioactive and immunoreactive material were lyophilized, reconstituted in 0.1% trifluoroacetic acid (TFA) and re-chromatographed on a Parcosil RP C4 column (250 Å, 20–45 μm, 20 × 125 mm); gradient from 30% acetonitrile, 0.1% TFA to 80% acetonitrile, 0.1% TFA in 100 min; flow rate, 3 ml/min, 2-min fractions (step 2). Again, aliquots of all HPLC fractions were assayed for bioactive and immunoreactive guanylin.

2.6. Detection of guanylin bioactivity

The increase in cyclic GMP content in human colon carcinoma cells (T84, passages 20–26; ATCC, Rockville, MD) was used as a sensitive detection system for bioactive guanylin as described [2].

2.7. Western blot analysis

For immunoblots, 5% aliquots (v/v) of all HPLC fractions were lyophilized and resuspended in sample buffer with 4% SDS (Merck, Darmstadt, Germany), 50 mM Tris-HCl, pH 8.45, 1 mM EDTA, 3.24 mM dithiothreitol (DTT; Roth, Karlsruhe, Germany), 12.5% glycerol (w/v) (Merck) and 0.002% Bromphenol blue (Merck) and incubated for 7 min at 95°C (reducing conditions). The samples were separated by tricine-SDS-PAGE in 17.5% gels [12]. The low M_W markers from Boehringer-Mannheim (Mannheim, Germany) were used for the M_W calibration. After electrophoresis, proteins were electroblotted onto hydrophobic polyvinylidene difluoride (PVDF)-based membranes (Pall, Dreieich, Germany). To block non-specific binding of the antibodies, blot strips were incubated in 5% skim milk in Tris-buffered saline containing 10 mM Tris and 150 mM NaCl (pH 8.0) with 0.05% Tween 20 (TBST). After washing in TBST the membranes were incubated overnight at 4°C with K39 antiserum (1:1,000). Immunoreactive proteins were visualized after incubation with alkaline phosphatase-conjugated goat antibody to rabbit IgG (1:8,000) (Sigma) using Nitro-blue tetrazolium and 5-bromo-4-chloro-3-indoylphosphate as chromogens (Sigma). Proteins remaining in the gels were fixed in 30% methanol and 10% acetic acid for 45 min before they were stained with Coomassic brilliant blue in 10% acetic acid for 1–2 h.

2.8. Plasma and urine extraction

Plasma samples were obtained from 30 healthy individuals (17 men, 13 women, aged 19–69 yrs, mean 47 yrs) and from 22 patients with renal insufficiency undergoing chronic hemodialysis (13 men, 9 women, aged 40–73 yrs, mean 62 yrs). 10 ml blood samples were withdrawn into ice-chilled tubes containing K_2EDTA and centrifuged at 2,500 × g for 20 min at 4°C. 2 ml aliquots of plasma were diluted 1:1 with 0.01 N HCl and adjusted to pH 3.0 with concentrated HCl. The samples were applied to Sep-Pak C18 cartridges (Waters) preactivated with methanol. The columns were washed with 0.01 N HCl and the absorbed materials were eluted with 2 ml of 30% 2-propanol/30% methanol/0.01 N HCl. 1 ml aliquots of the eluents (corresponding to 1 ml plasma) were lyophilized and reconstituted for RIA and bioassay. Urine samples (5 ml) of 6 healthy volunteers were extracted with Sep-Pak C18 cartridges following the same procedure as detailed for plasma.

2.9. Statistics

Statistical differences between mean values were determined by analysis of variance followed by the Fisher protected least significant difference test for comparison of different means. P values of less than 0.05 were considered significant.

3. Results

A standard curve produced by a plot of the percentage of bound counts (B) divided by the zero standard (B_0) vs. the log concentration of guanylin-25–37 is shown in Fig. 1. The minimum detectable quantity of guanylin-25–37 was 3.1 fmol/tube (96% displacement). The binding of [^{125}I](Tyr^{24})-guanylin-25–37 in the absence of unlabelled guanylin-25–37 (zero standard, B_0) was 26 ± 1.4% (n = 8 assays). The concentration producing 50% inhibition of binding was 200 fmol/tube. The intra- and inter-assay coefficients of variation were 6 and 14%, respectively. The antiserum did not show any cross-reaction with other known cyclic GMP-enhancing peptides (CDD/ ANP-99–126, BNP, C-type natriuretic peptide and urodilatin) nor with other peptide hormones such as angiotensin I and II, endothelin-1, pituitary adenylate cyclase activating peptide (PACAP)-27 and PACAP-38, vasoactive intestinal peptide (VIP), neuropeptide Y, neurokinins A and B, substance P, neurotensin, bradykinin or bombesin. The maximum concentration tested to reveal cross-reactivity of these peptides was 1 nmol/tube (10 μmol/l).

To characterize the specificity of the RIA further, circulating guanylin was purified from human blood he-

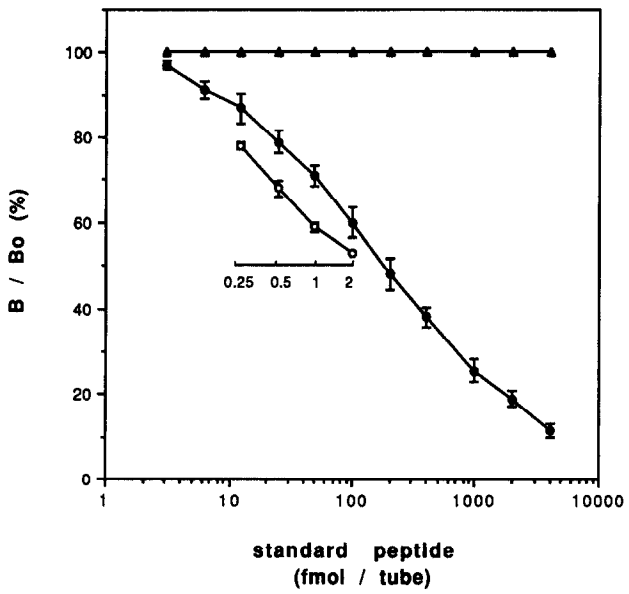

Fig. 1. Radioimmunoassay (RIA) for circulating human guanylin. Standard curve for guanylin-25–37 (●, mean ± S.E.M., $n = 8$) and crossreactivities with natriuretic peptides (CDD/ANP-99–126, BNP, CNP, urodilatin: ▲). Dilution curve of normal human plasma extract (○); serial dilutions of original plasma volume are denoted under the curve. The results are expressed as the percentage of bound counts (B) divided by the zero standard (B_0).

mofiltrate using nearly identical conditions as for its initial isolation [2]. After extraction of polypeptides with the alginic acid method of Mutt–Forssmann [10,11], the material was chromatographed on a preparative RP C4 column (step 1). Fraction aliquots were tested in the T84 cell bioassay and in the RIA. Representative HPLC profiles of blood hemofiltrate extract are shown in Fig. 2. The guanylin- RIA reactivity in the fractions showed two distinct peaks. The major component eluted in the position of guanylin-22–115, between 83 and 88% buffer B (elution time for guanylin-22–115 known from the isolation procedure [2]). The minor component eluted earlier, between 66 and 73% buffer B (step 1, Fig. 2). Both components led to marked increases in cyclic GMP content of T84 cells (Fig. 2). Fractions containing guanylin-22–115 (fractions 15–18) were re-chromatographed on a semi-preparative RP C4 column. Again, bioactive and immunoreactive guanylin co-eluted in the same fractions between 53 and 58% buffer B (step 2, Fig. 2).

In each bioassay the cyclic GMP response of T84 cells to the hemofiltrate fractions was compared to their response to synthetic guanylin-101–115 (1 nM–10 μM) (Fig. 2). The HPLC fractions exhibited corresponding bioactivity with respect to their concentrations of IR-guanylin. This correlation was already obtained in the second HPLC step. As an example, in this step, fraction 30 contained the highest concentration of bioactive as well as immunoreactive guanylin. The concentration of IR-guanylin was 34.8 pmol/ml (34.8 nM). In the bioassay, 1 ml of fraction 30 evoked a 27-fold increase in cyclic GMP content of T84 cells. For comparison, in the same experiment, 100 nM synthetic guanylin-101–115 induced a 44-fold increase in cyclic GMP content of T84 cells (Fig. 2).

In parallel experiments, aliquots of all HPLC fractions were separated by tricine-SDS-PAGE and immunoblotted. The tricine electrophoresis system was preferred to conventional SDS-PAGE because of its higher resolution of low M_W proteins, especially in the range between 5 and 20 kDa [12]. Western blot analyses showed that the peptide predominantly recognized by the K39 antiserum was in the range of 10–11 kDa (M_W of circulating human guanylin, 10.3 kDa). The main immunoreactive peptide was present only in lanes loaded with HPLC fractions

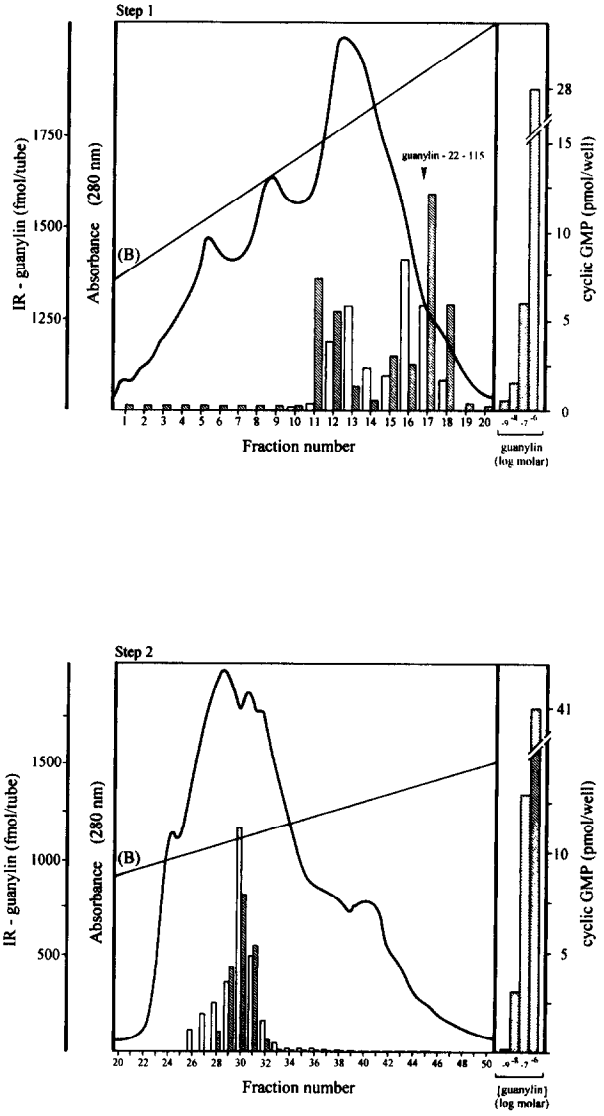

Fig. 2. Representative chromatograms obtained during purification of human guanylin from hemofiltrate. HPLC conditions are given in section 2. (B), eluent B (%, v/v). Columns indicate the immunoreactive and bioactive fractions (open columns, RIA; hatched columns, bioassay). At the right of the graphs is given the concentration-dependent stimulation of cyclic GMP content in T84 cells by rat guanylin-101–115 (guanylin: 1 nM–10 μM).

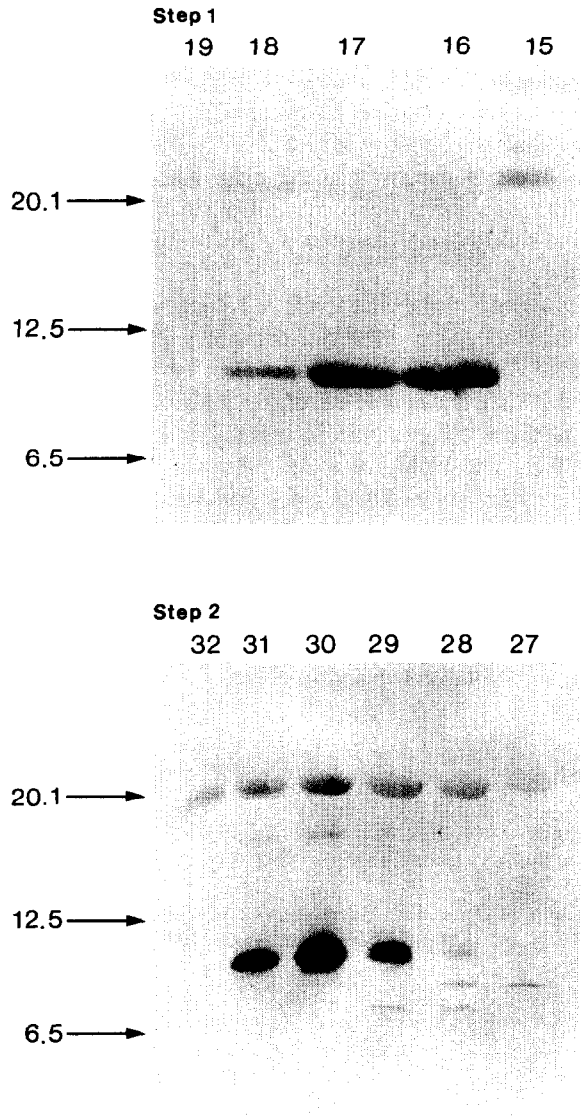

Fig. 3. Western blots after tricine-SDS-PAGE of HPLC-fractionated human hemofiltrate (steps 1 and 2). Positions of fraction aliquots are indicated on the top. Immunostaining was with K39 antiserum against guanylin-25–37 (1:1,000). The migration positions of molecular weight markers (Boehringer-Mannheim) are shown on the left of the blot (20.1, trypsin inhibitor; 12.5, cytochrome *c*; and 6.5, aprotinin). Note the prominent immunoreactive band below 12 kDa in the lanes corresponding to fractions 16–18 (step 1) and fractions 29–31 (step 2).

containing guanylin according to the bioassay and RIA (step 1, fractions 16–18; step 2, fractions 29–31) (Fig. 3). The identity of this main immunoreactive band as guanylin-22–115 was further suggested from the fact that the same peptide band (in identical HPLC fractions) was labeled by other guanylin antisera directed against a midportion (positions 13–25: antiserum K42) and against the C-terminal end (antiserum K605) of circulating human guanylin (data not shown; for details of the antisera, see [5]). In addition, the immunoblot with K39 antiserum showed minor immunoreactive bands above 20 kDa. These weakly stained bands were also present in HPLC fractions that did not contain measurable guanylin neither by bioassay nor by RIA, suggesting that they were due to non-specific cross-reactions of the K39 antiserum in the immunoblot which, however, do not interfere in the RIA.

The RIA was applied to measure guanylin concentrations in Sep-Pak-extracted human plasma. The dilution curves generated from human plasma extracts paralleled that of standard guanylin-25–37 (Fig. 1). The mean concentration of IR-guanylin in plasma of 30 healthy subjects was 42 ± 3.1 fmol/ml (mean ± S.E.M. range, 25–66 fmol/ml). In plasma collected from 22 patients undergoing dialysis because of severe renal insufficiency, levels of IR-guanylin were significantly increased up to 1,074 ± 24 fmol/ ml ($P < 0.05$).

Resting cyclic GMP content of T84 cells in the presence of isobutylmethylxanthine (IBMX, 1 mM) was 362 ± 23 fmol/well ($n = 4$) 60-min incubation of T84 cells with the 0.4 ml plasma extracts of healthy subjects ($n = 11$) increased cyclic GMP content to 480 ± 60 fmol/well (not significant). In contrast, the 0.4 ml plasma extracts of dialysis patients ($n = 12$) led to 3–7 fold increases in cyclic GMP content of T84 cells (1,760 ± 360 fmol/well, $P < 0.05$). We were also able to detect IR-guanylin in urine. The mean concentration of IR-guanylin in urine extracts of six healthy subjects was 95 ± 18 fmol/ml. The urine extract (0.4 ml; incubation time, 60 min) induced 7–12 fold increases in cyclic GMP content of T84 cells (3,390 ± 530 fmol/well, $n = 6$, $P < 0.01$).

4. Discussion

A sensitive RIA for the main circulating molecular form of human guanylin has been developed. A minimal concentration of 3.1 fmol/tube can be detected, thus enabling us to measure IR-guanylin in human plasma. The antigen for immunization was a peptide containing positions 4–16 of the circulating hormone. Immunization with a MAP was chosen because the conventional step of conjugation of peptide to carrier can be omitted and because of the higher immunogenicity of MAPs in comparison to the corresponding monomeric peptide [7]. The antiserum used for RIA (K39) recognizes guanylin–MAP (Western blot, not shown), monomeric guanylin-25–37 (ELISA, RIA) and, more importantly, native circulating human guanylin-22–115 (Western blot). It shows no crossreaction with a large series of other circulating hormones tested, in particular with other cyclic GMP-stimulating peptides.

After extraction of polypeptides from blood hemofiltrate and subsequent fractionation by RP-HPLC, the assay of the obtained fractions for immunoreactive guanylin correlates with the detection of cGMP-stimulating material in the T84 cell bioassay. With both assays gua-

nylin-22–115 is detected in the same fractions and in comparable concentration ranges. However, in contrast to the bioassay, the RIA is more sensitive and allows the exact quantification of guanylin. Its sensitivity is more than two orders of magnitude higher than that of the bioassay (detection limits: RIA, 3 fmol/i.T.; bioassay, 1 pmol/well). In the first RP-HPLC step of blood hemofiltrate, besides guanylin-22–115 an additional peak of bioactive and immunoreactive material was detected. Further HPLC purification steps confirmed the correspondence between RIA and bioassay also for this second component (data not shown). It should be emphasized that in this context other known guanylyl cyclase-stimulating peptides (CDD/ANP-99-126, BNP, CNP, Urodilatin) do not interfere either with the RIA or the bioassay (i.e. these peptides do not alter cyclic GMP content of T84 cells). Therefore, we postulate that a second hormonal form of guanylin circulates in human blood. Presently, investigations are being carried out to characterize its precise molecular identity.

Application of this RIA allows for the first time the detection of guanylin in human plasma. Levels of immunoreactive guanylin in plasma of healthy subjects are in the range of 25–66 fmol/ml, i.e. far below the concentrations that can be detected by the T84 cell bioassay. In our institute, blood hemofiltrate obtained from renal-insufficient patients is an established source for the isolation of human plasma peptides. Actually, circulating human guanylin was isolated from this source [2]. For that reason we were interested in the evaluation of guanylin plasma levels in renal insufficient patients. In all patients, plasma concentrations of IR-guanylin are increased 10–20 fold (range: 0.5–2 nM/l). The guanylin bioassay confirms this observation: extracted plasma of these patients causes a 3–7 fold increase in cyclic GMP content of T84 cells. Similar increases are induced by 0.6–1 nM/l guanylin-101–115.

Enhanced levels of IR and bioactive guanylin in plasma of dialysis patients indicate that the kidneys are involved in the metabolism and/or elimination of the hormone. In fact, we also detect guanylin in urine. However, in urine, guanylin bioactivity is disproportionately high compared to concentrations of IR-guanylin. Published data suggest that the C-terminal end of the hormone carries the active (GC-C stimulating) domain [1]. A possible explanation for our findings is that circulating guanylin-22–115 is filtered and metabolized (proteolized) in the kidney, and therefore urine contains bioactive C-terminal fragments of guanylin that cannot be detected by RIA. Recently, uroguanylin, a guanylin homologue peptide, was isolated from opossum urine [13]. Although the presence of this peptide in urine from other species has not been described until now, we cannot exclude the possibility that human urine may contain other guanylyl cyclase C-stimulating peptides that escape detection by the presented RIA.

Despite the enhanced plasma levels of guanylin, none of the dialysis patients included in our study had diarrhea or other gastrointestinal disorders. This suggests that the hormone, once circulating in blood does not reach its intestinal receptor at the luminal side of the enterocytes.

In ongoing studies the levels of IR-guanylin in patients with several gastrointestinal disorders, especially those patients with intestinal diarrhea, are being investigated further. Also, a major focus of our studies is the comparison between IR-guanylin in plasma and intestinal fluid secretion. The results will indicate whether circulating IR-guanylin levels reflect intestinal release, and hopefully will contribute to a clarification of the pathophysiological role of this hormone in disturbances of intestinal electrolyte transport.

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